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Effect of balneotherapy in sulfurous water on an in vivo murine model of osteoarthritis

Carlos Vaamonde-García[1], Ángela Vela-Anero[1,2], Tamara Hermida-Gómez[3], Elena Fernández-Burguera[2,3], Purificación Filgueira-Fernández[3], Noa Goyanes[3], Francisco J. Blanco[4] & Rosa Meijide-Faílde[1]

1 Facultad de Ciencias de la Salud, Grupo de Terapia Celular y Medicina Regenerativa, Agrupación Estratégica CICA-INIBIC, Complexo Hospitalario Universitario A Coruña, Campus de Oza, 15006, A Coruña, Spain
2 CIBER-BBN, Barcelona, Spain
3 Grupo de Investigación en Reumatología (GIR), Agrupación Estratégica CICA-INIBIC, Complexo Hospitalario Universitario A Coruña, Sergas, Universidad de A Coruña, As Xubias, 15006, A Coruña, Spain
4 Grupo de Investigación de Reumatología, INIBIC-Hospital Universitario A Coruña, Departamento de Fisioterapia, Medicina y Ciencias Biomédicas, Universidad de A Coruña, A Coruña, Spain

Abstract
Osteoarthritis (OA) is a chronic joint disease that results in progressive cartilage destruction and subsequently joint dysfunction. Growing evidence indicates beneficial impact of balneological interventions in OA; however, their mechanisms of action are still unclear. Here, we evaluate the effect of balneotherapy in sulfurous water in an OA experimental model. Experimental OA was induced in Wistar rats by transection of the medial collateral ligament and removal of the medial meniscus of the left knee. Animals were randomized into three groups: non-treated (control) and balneotherapy using sulfurous water (SW) or tap water (TW). Macroscopic evaluation was performed, as well as evaluation of pain levels and analysis of motor function by rotarod test. Histopathological changes in articular cartilage and synovium were also evaluated. The presence of matrix metalloproteinase-13 (MMP-13) and oxidative damage markers was assessed by immunohistochemistry. Joint destabilization induced joint thickening, loss of joint flexion, and increased levels of pain. At day 40, animals from SW group presented lower pain levels than those from control group. Experimental OA also affected motor function. Balneotherapy in sulfur-rich water significantly improved joint mobility in relation to that in tap water. Besides, we observed that cartilage deterioration was lower in SW group than in the other two groups. Likewise, SW group showed reduced levels of MMP-13 in the cartilage. Conversely, we failed to observe any modulation on synovial inflammation. Finally, balneotherapy in sulfurous water diminished the presence of oxidative damage markers. Our results suggest the beneficial effect of balneotherapy in sulfur-rich water on an experimental model of OA, showing a reduced cartilage destruction and oxidative damage. Thus, these findings support the use of balneotherapy as a non-pharmacological treatment in OA.

Keywords Balneotherapy, hydrogen sulfide, osteoarthritis, oxidative damage, cartilage

Introduction

Osteoarthritis (OA) is the most common chronic disease of the joints, which presents with loss of function and severe pain (Kraus et al. 2015). OA is characterized by degeneration of cartilage and its underlying bone, as well as by synovial inflammation that are directly linked to clinical symptoms (Kraus et al. 2015). Thus, although its etiology is still unclear, it is widely accepted that a shift of cartilage metabolism toward upregulated catabolic activities and synovitis could contribute to OA development (Kraus et al. 2015; Bolduc et al. 2019).

A growing number of evidence indicates that reactive oxygen species (ROS) production is increased in OA causing oxidative stress which may participate in the pathogenesis of this disease. Likewise, by-product of oxidative damage to proteins, lipids, or DNA strongly promotes the activation of pathological pathways in the articular tissues, such as inflammatory and destructive processes (Biniecka et al. 2011a, b; Bolduc et al. 2019). Increased pro-inflammatory responses in the synovium and cartilage contribute to cartilage destruction that in turn amplifies joint inflammation, creating a vicious circle that favors development of joint disorders like OA (Bultink et al. 2012; Robinson et al. 2016; Vaamonde-García and López-Armada 2019). For instance, inflammatory mediators like cytokines and adipokines could increase the synthesis of matrix metalloproteinases (MMPs), well-characterized enzymes involved in the degradation of the extracellular matrix of the cartilage (Cheleschi et al. 2018; Zhang and Zhao 2018; Yang et al. 2017). One of the most important enzymes implicated in collagen type II degradation is collagenase 3 (matrix metalloproteinase-13; MMP-13). The overexpression of this enzyme triggered by oxidative stress and inflammation (Waldron et al. 2018; Kar et al. 2010) is a pivotal event in the development of OA; it has been suggested that MMP-13 inhibition may be a putative strategy to target disease progression (Burrage et al. 2006). Nonetheless, synovial tissue may also contribute to OA pathogenesis, as relevant changes in this tissue can be observed even before cartilage degeneration has been detected. These changes are characterized by thickening of the synovial lining layer, altered cell density of synovial stroma, and infiltration of inflammatory cells (Mathiessen and Conaghan 2017), thereby multiple studies indicate a clear relationship between synovitis and OA symptoms and development (Mathiessen and Conaghan 2017; Scanzello and Goldring 2012).

There is currently no cure for OA and existent treatment consists of relieving the pain, targeting the disease progression with non-pharmacological and pharmacological treatments, and finally joint replacement. In relation, balneotherapy is widely used as a complementary non-pharmacological treatment for musculoskeletal disorders, as it has shown protective effect against pain and joint malfunction (Antonelli et al. 2018). Balneotherapy is mainly based on the use of thermal mineral waters rich in therapeutically active components, like natural gases as CO_2 or sulfur. In this sense, the effects of sulfurous mineral waters have been associated to the presence of sulfur mainly in the form of hydrogen sulfide (H_2S). H_2S is a gasotransmitter and recognized endogenous mammalian mediator (Szabo 2018). This gas is involved in signaling in different systems presenting a wide range of biological properties. In this regard, the presence of this gas and the necessary machinery for its synthesis in the joint are currently accepted (Fox et al. 2012; Whiteman et al. 2010). Besides, different in vitro findings indicate that H_2S at low concentrations exerts anti-inflammatory and anti-oxidant actions in articular cells (Fox et al. 2012; Whiteman et al. 2010; Burguera et al. 2014; Vela-Anero et al. 2017).

A growing number of evidence suggests beneficial impact of balneotherapy in OA patients; however, more studies are still necessary to determine to what extent the different components presented in the waters could influence in its effects (Antonelli et al. 2018). In this sense, sulfurous mineral waters have been traditionally used in medical hydrology as treatment of musculoskeletal disorders (Carbajo and Maraver 2017), although their mechanisms of action in vivo have not been completely elucidated yet. Thus, in the current study, we evaluate the effects of balneotherapy in sulfurous water on induced pain, loss of mobility, and cartilage degradation in an experimental model of OA in rats, as well as on activation of catabolic and oxidative pathways.

Material and methods

Experimental osteoarthritis in rats

All animal experiments were performed according to protocols approved by the Local Ethical Committee of Animal Experimentation and European Directive 2010/63. Eighteen female Wistar rats (Harlan Interfauna Ibérica, Barcelona, Spain) weighing between 350 and 450 g (8–10 months) were used. The animals were kept at room temperature (20–24 °C) and commercial food and water were available ad libitum. Experimental osteoarthritis was induced by transection of the medial collateral ligament and removal of the medial meniscus of the left joint (Caramés et al. 2012). The right joint was sham-operated and employed as control. Then, animals were randomized into three groups (6 rats per group): Non-treated (control), balneotherapy using sulfurous water (SW), and balneotherapy using tap water (TW). Thermal water was obtained from Spa of Carballo (Spain), a sulfurous water of weak mineralization. Physicochemical characteristics of employed waters are shown in Table 1. Balneotherapy was performed by placing the rats in hot water (37 °C) for 20 min, 5 days per week, from day 7 after surgery to day 40. Animals were euthanized at day 40.

Table 1 Physicochemical characteristics of waters employed in the study. Mineral water was obtained from Spa of Carballo (Spain) and analyzed by Geological and Mining Institute of Spain

	Mineral wáter[1]	Tap water[2]
pH value	8.8	7.2
Electrical conductivity (μS/cm)	655	124.3
Total mineralization 180 °C (mg/L)	388	59.3
Hardness (mg $CaCO_3$/L)	10.1	22.77
Alkalinity (mg $CaCO_3$/L)	120	23.29
Cations		
Sodium (Na+) (mg/L)	132	12.67
Potassium (K+) (mg/L)	5	1.06
Calcium (Ca^{+2}) (mg/L)	3.8	4.03
Magnesium (Mg^{+2}) (mg/L)	0.2	3.07
Silica (SiO_2) (mg/L)	67	NA
Anions		
Fluoride (F^-) (mg/L)	20.2	< 0.15
Chloride (Cl−) (mg/L)	44.1	16.01
Bicarbonate (CO_3^{-2}) (mg/L)	146.4	25.78
Sulfate (SO_4^{-2}) (mg/L)	76.4	7.4
Nitrate (NO3−) (mg/L)	< 0.5	6.3
Nitrite (NO2−) (mg/L)	< 0.005	0.05
Sulfide (HS^{-1}) (mg/L)	20.3	0
Gas		
Carbon dioxide (CO_2) (mg/L)	1.1	0
Hydrogen sulfide (mg/L)	0.2	0

1Dirección General de Minas. Xunta de Galicia
2Tap water was obtained from Cecebre reservoir (Spain) and analyzed by Servicios de Apoyo a la Investigación (University of A Coruña)

Quantification of H2S

Levels of H2S in water was assayed by an ion-selective microelectrode (Lazar Research Lab. Inc., USA, model LIS-146GSCM) attached to a voltage meter (Model 6230N, Jenco Electronics, LTD, Taiwan). An anti-oxidant buffer stock solution was prepared with sodium salicylate, ascorbic acid, and NaOH in distilled water, following the electrode instructions. In order to prepare the working solution, the stock solution was further diluted 1:3 in distilled water. Water sample (200 μL) was mixed 1:1 with the working solution, sealed with Parafilm M®, and incubated at 37 °C for 1 h. Subsequently, the microelectrode was immersed in the fluid, and the voltage value was recorded and converted to H2S concentration with a calibration curve prepared with Na2S standard.

Macroscopic clinical evaluation

Macroscopic evaluation of the animals was performed at days 0 (before surgery), 7, 15, and 40. Firstly, loss of weight was monitored and the articular diameter was measured by a digital caliper (S-CalWork, Sylvac, Malleray, Switzerland) in order to evaluate joint thickening. Evaluation of pain levels was tested by palpation of the knee and subsequently scoring the response of the rats to the joint manipulation on the 0 to 2 scale, being 0 no response and 2 the most painful response. Similarly, joint crepitation was scored from 0 to 2 (no crepitation to marked crepitus). Finally, loss of joint flexion and extension angle were measured by a protractor.

Rotarod performance test

All the animals were subject to rotarod performance test (Ugo Basile S L, Varese, Italy) at days 0 (before surgery), 7, 15, and 40 after a training period of 2 weeks. Briefly, rats were placed on a cylinder (rod) rotating at 30 rpm for 300 s and forced to move. Then, the number of falls during this period was registered as well as the time that rats stayed on the rotarod before the first fall.

Histological analysis

Knee joints from rats were dissected, fixed in 4% formaldehyde in phosphate buffered saline (PBS), decalcified with DECALTM (Histolab, Askim, Norway), and embedded in paraffin (frontal section). Lesions in the synovial tissue and cartilage were evaluated by semi-quantitative analysis by two blinded researchers using an Olympus microscope (Olympus BX61, Olympus Biosystems, Barcelona, Spain). Sections (4-μm thick) of the joint were stained with hematoxylin and eosin, Masson's trichrome, or Safranin-O-fast green (Merck, Madrid, Spain). Two samples per animal were employed to evaluate damage score for cartilage and synovial tissues. According to the semi-quantitative-modified OARSI score, the grade of the cartilage lesion was scored from 0 to 6 (0 being not damaged and 6 most damaged) (Glasson et al. 2010). The grade of the synovial lesion was scored from 0 to 9 (0 being not damaged and 9 most damaged), and the evaluation parameters were numbers of lining cell layers, proliferation of the subintima tissue, and infiltration of inflammatory cells (Krenn et al. 2006).

Immunohistochemistry

MMP-13, 8-hydroxy-2′-deoxyguanosine (8-oxo-dG), and 4-hidroxi-2-nonenal (4-HNE) levels were evaluated on sections from paraffin-embedded joints of rats. Slides were first deparaffinized with xylene and then rehydrated in graded ethanol and water. Heat-mediated antigen retrieval was performed in citrate buffer (pH 6.0; Dako, Glostrup, Denmark) or ethylenediaminetetraacetic acid (EDTA) buffer (pH 9.0; Dako) for MMP-13 and 4-HNE detection respectively. Endogenous peroxidase activity was quenched using a commercial reactive (Dako) for 10 min. After washing in PBS, slides were incubated 1 h at room temperature with anti-8-oxo-dG (1:200, Abcam, Cambridge, UK) or anti-4-HNE (1:150, Abcam) antibodies, or anti-MMP-13 (1:100, Abcam) after blocking with 20% normal goat serum for 10 min. Bound antibodies were detected with a goat secondary antibody (ready-to-use; Dako) and diaminobenzidine using the commercial EnVision™ Detection System (Dako). Finally, sections were counterstained with Gill III hematoxylin (Merck) and mounted with DePeX (Sigma). Multiple images of each slide (two slides per animal) were captured with a computer-controlled digital camera (Olympus BX61, Olympus). By using an image processing software (Image J software, http://imagej.nih.gov/), the percentage of chondrocytes presenting a positive signal of immunostaining in the cartilage was measured and represented as the ratio positive cells in the cartilage.

Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM) or as representative results, as indicated. The GraphPad PRISM version 5 statistical software package (La Jolla, CA, USA) was used to perform one-way analysis of variance followed by Bonferroni's post hoc comparison test. Statistically significant differences between experimental groups were determined by unpaired comparison test. $P < 0.05$ was considered statistically significant.

Results

Balneotherapy in sulfurous water attenuates joint pain and protects against motor dysfunction after experimental OA

High levels of sulfide ion in the spring water used in this study were confirmed by an ion-selective microelectrode, detecting similar content (24,23 mg/l) than that observed for this spring in previous studies (Table 1 and Maraver and Armijo 2010). Orthopedic examination and joint goniometry were carried out in order to evaluate the effect of balneotherapy in sulfurous water on joints with surgically induced lesions. As expected, experimental OA scarcely altered weight of the rats throughout the model (Fig. 1a). Animals showed joint thickening at day 7 after surgery that was significantly recovered at day 40 in all experimental groups (Fig. 1b). Strikingly, joints from the control group presented at day 40 lower articular diameter than at day 0 before surgery, suggesting a loss of muscle mass in these rats. Additionally, we observed that the surgical destabilization of the joint induced at day 7 showed a significant level of pain in all experimental groups (Fig. 1c). However, rats under SW treatment displayed at day 40 lower pain levels than those from the control group, which was not observed in the TW group. The presence of articular crepitation was also analyzed, detecting initial crepitus in the joint from all the tested animals. Interestingly, the control group maintained the significant levels of crepitation at day 40, whereas rats from balneotherapy groups showed non-statistical differences in relation to day 0 (Fig. 1d). Similarly, joint flexion was affected along the tested times in the non-treated groups, but loss of joint flexion was only transient in the SW group (Fig. 1e). Conversely, we failed to observe loss of joint extension after surgical destabilization (Fig. 1f).

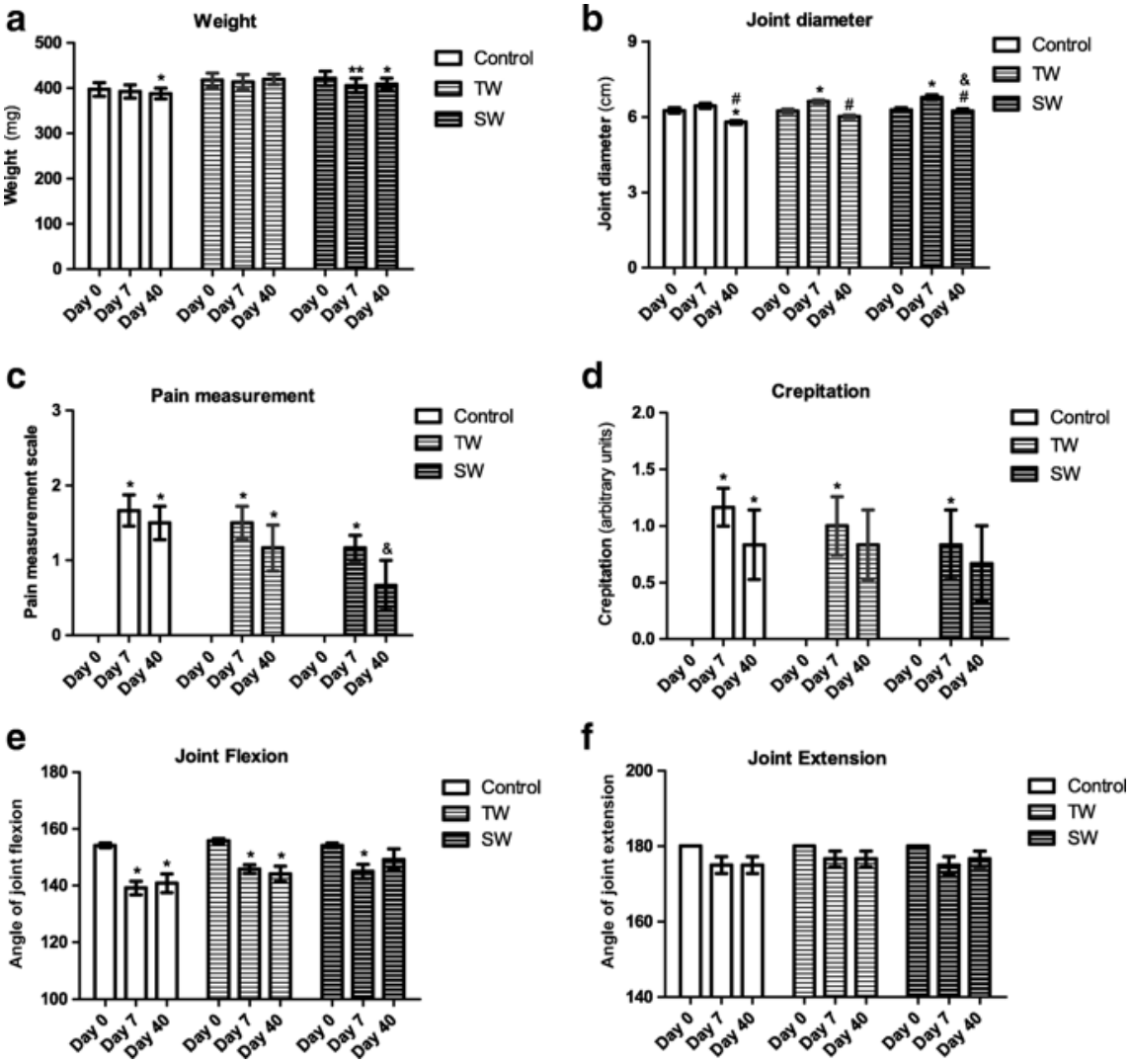

Fig. 1 Orthopedic examination and joint goniometry. Macroscopic evaluation of the animals from the experimental groups (non-treated (control) and balneotherapy in tap water (TW) or in sulfurous water (SW)) was performed at days 0 (before surgery), 7, 15, and 40. Firstly, weight (**a**) and articular diameter (**b**) were monitored. Evaluation of pain levels (**c**) and joint crepitation (**d**) angle of joint flexion (**e**) and extension (**f**) were measured by arbitrary scales as previously indicated. Finally, angle of joint flexion and extension over the course of the model was assessed by a protractor. Values are mean ± SEM ($n = 6$ independent animals for each condition). $^{*}P \leq 0.05$ vs. day 0; $^{\#}P \leq 0.05$ vs. day 7; $^{\&}P \leq 0.05$ vs. respective day in control group

We also analyzed the motor function in the animals under experimental OA by rotarod performance test. Joint destabilization triggered an initial increment of number of falls in the rotarod in all the experimental groups (Fig. 2a). The SW group showed at day 15 a significant recovery compared with that observed in the TW group. Likewise, we also detected in the SW group that number of falls at day 15 and 40 were not significantly different than that registered at day 0 (Fig. 2a), suggesting a stable recovering. Additionally, the time that the rats stayed on the rotating cylinder before the first fall dropped after surgical intervention (at day 7) in all the experimental groups (Fig. 2b). Interestingly, animal treated with balneotherapy in sulfurous water showed at 15 days longer stay on the rotarod than balneotherapy control, but non-significant differences were detected in relation to the control group (Fig. 2b).

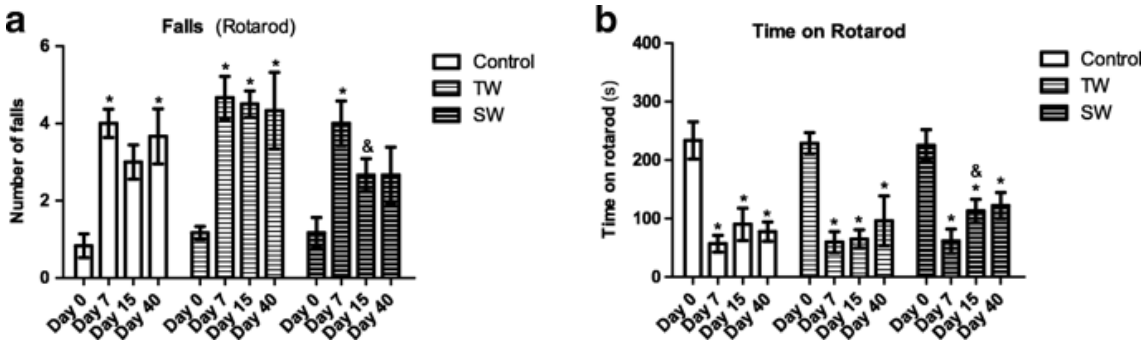

Fig. 2 Analysis of the articular motor function by rotarod performance test. Animals from the three experimental groups were forced to move in the rotating cylinder for 300 s. The number of falls (**a**) during this period was monitored, as well as the time remaining on the rotarod (**b**). Values are mean ± SEM ($n = 6$ independent animals for each condition). $^{*}P \leq 0.05$ vs. day 0; $^{\&}P \leq 0.05$ vs. respective day in the TW group. Control, non-treated; TW, balneotherapy in tap water; SW, balneotherapy in sulfurous water

Balneotherapy in sulfurous water protects against cartilage destruction induced by joint surgical destabilization

Histopathological alterations in the cartilage and synovial tissue were analyzed by conventional staining methods. Cartilage from the medial compartment of the knee under surgical destabilization showed loss of Safranin-O staining, indicator of proteoglycan content, and presence of vertical clefts/erosion whose extension varied between experimental groups (Fig. 3a). The semi-quantitative modified OARSI score confirmed these differences, observing that the articular cartilage destruction in the medial compartment was lower in the joints from the SW group than in those from the control group (Fig. 3d), achieving statistical differences in the tibial plateau in relation to that observed in the control group and in both tibial plateau and femoral condyle in comparison with that observed in the TW group (Fig. 3b). Conversely, non-significant alterations were detected in the cartilage from lateral compartment as expected (Fig. 3c).

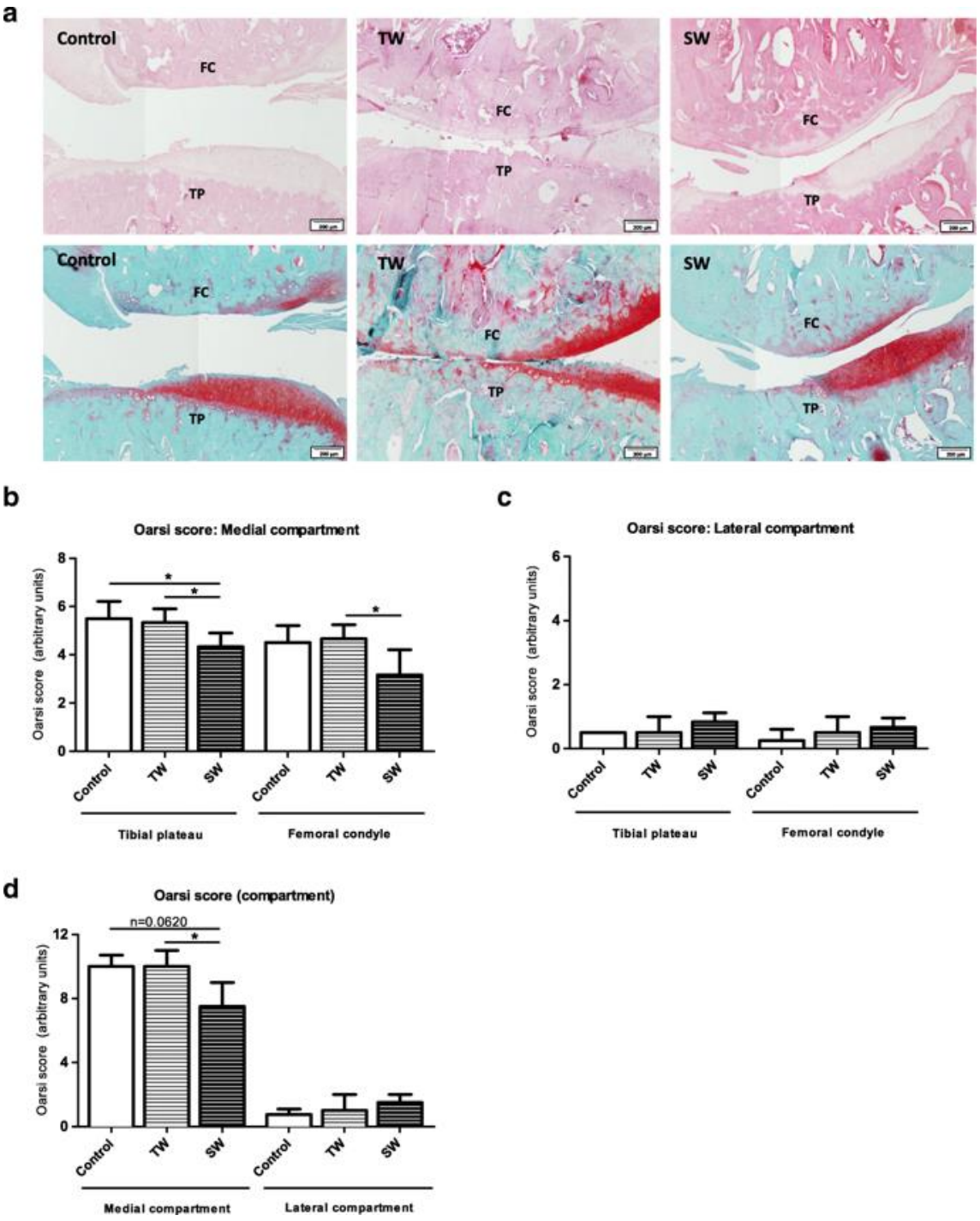

Fig. 3 Histological analysis of articular cartilage. Cartilage lesions were evaluated by the semi-quantitative modified OARSI score, as previously indicated in the "Material and methods" section. **a** Representative images of joint sections stained with hematoxylin and eosin (above) and Safranin-O-fast green (below) from each group of study, showing cartilage from tibial plateau (TP) and femoral condyle (FC) of medial compartment. Analysis of semi-quantitative score of pathological alterations in the cartilage from medial (**b**) and lateral (**c**) compartments, as well as the sum in both compartments (**d**). Values are mean ± SEM ($n = 3$ independent animals for each condition). *$P \leq 0.05$. Control, non-treated; TW, balneotherapy in tap water; SW, balneotherapy in sulfurous water

The presence of pathological alterations in the synovial tissue was assessed by the semi-quantitative Krenn score (Fig. 4). We registered a clear increase in the number of lining cell layers (Fig. 4b), a slight proliferation of subintima tissue (Fig. 4c), and limited cell infiltration in the synovium from all experimental groups (Fig. 4d). However, non-significant differences were found among the three groups (Fig. 4f).

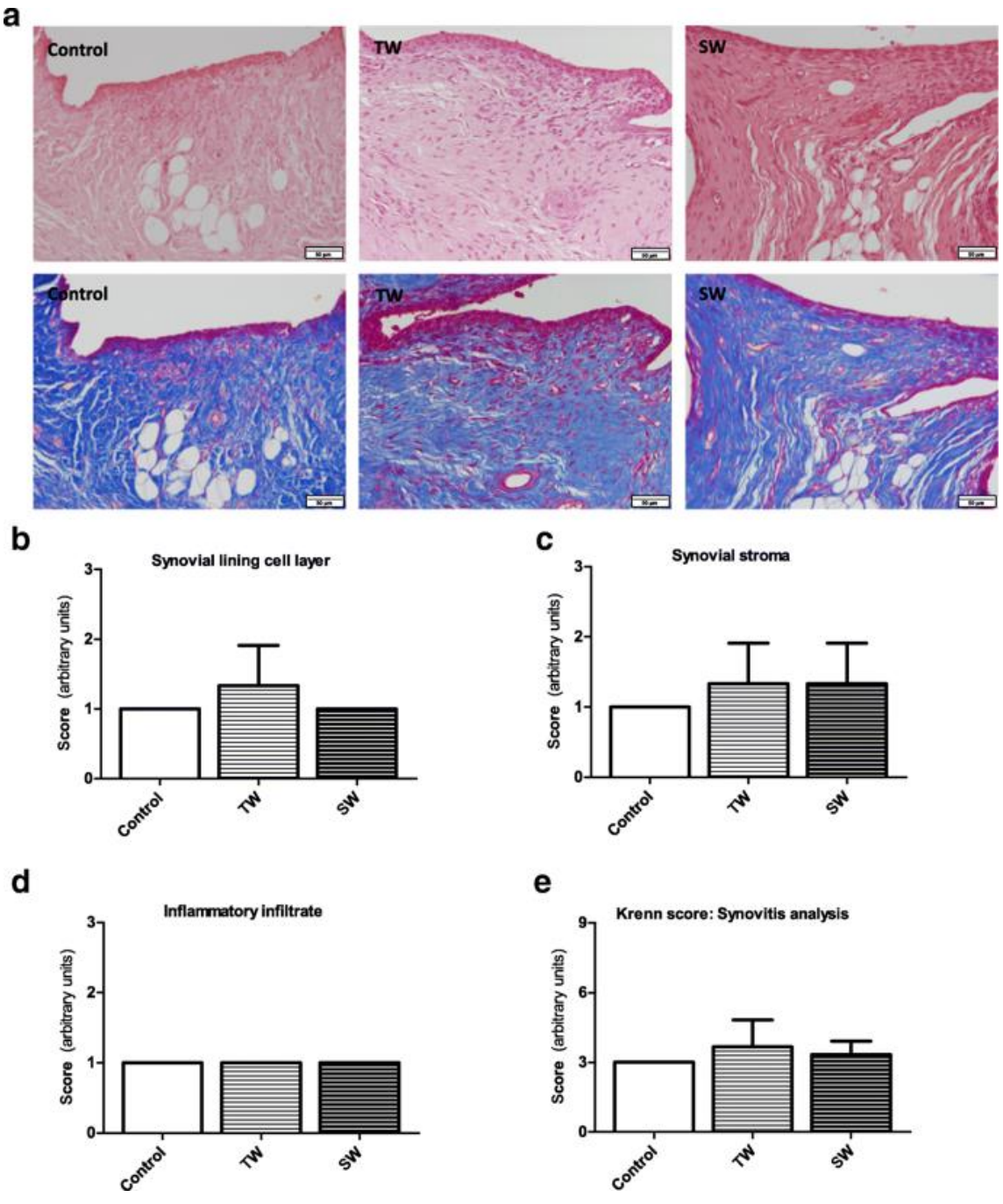

Fig. 4 Histological analysis of synovial tissue. Pathological responses in the synovial tissue were evaluated by semi-quantitative Krenn score as previously indicated in the "Material and methods" section. **a** Representative images of synovial sections stained with hematoxylin and eosin (above) and Masson's trichrome (below) from each group of study. Analysis of semi-quantitative score of the following pathological alterations in the synovial tissue: Number of lining cell layers (**b**), proliferation of the subintima tissue (**c**), and infiltration of inflammatory cells (**d**). **e** The sum of the pathological changes in the tissue is shown. Values are mean ± SEM ($n = 3$ independent animals for each condition). No significant differences were found. Control, non-treated; TW, balneotherapy in tap water; SW, balneotherapy in sulfurous water

In order to elucidate the molecular mechanisms responsible for the protective effect of balneotherapy in sulfurous water on cartilage destruction, we analyzed by immunohistochemistry the expression of MMP-13, one of the main enzymes involved in the ECM degradation, in the cartilage from the control group (Fig. 5a) and the SW group (Fig. 5b). We did not include the SW group in the analysis since non-significant differences were observed between non-treated and treated with balneotherapy in tap water. As observed in Fig. 5 c, cartilage from the control group showed a significant higher number of cells positive for MMP-13 than in the tissue from the SW group

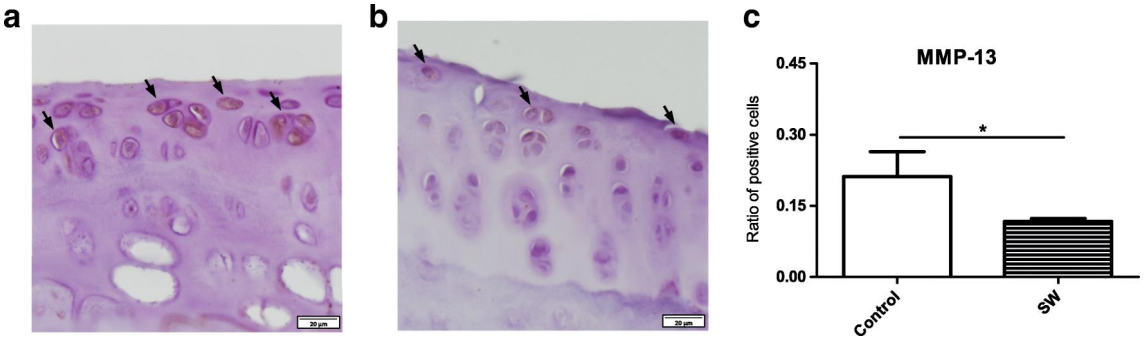

Fig. 5 MMP-13 expression in the articular cartilage. Representative samples of MMP-13 immunohistochemistry in the cartilage from control (**a**) or balneotherapy in sulfurous water group (**b**). Arrows indicate positive cells for immunostaining. **c** Quantitative analysis of MMP-13-positive cells. Values are mean ± SEM ($n = 3$ independent animals for each condition). *$P \leq 0.05$. Control, non-treated; SW, balneotherapy in sulfurous water

Oxidative damage is modulated by balneotherapy in sulfurous water

ROS overproduction may cause oxidative damage to DNA, proteins, and lipid membranes. Likewise, a growing number of evidence supports the pivotal role of oxidative damage in chronic arthropathies like OA (Sellam and Berenbaum 2010; Robinson et al. 2016). For this reason, we analyzed the presence of oxidative damage in the cartilage by immunohistochemistry of 8-oxo-dG, an indicator of DNA damage, and 4-HNE, a maker of membrane lipid oxidation. As shown in Fig. 6 a, experimental OA elicited the production of 8-oxo-dG in the joint. Interestingly, cartilage from the SW group presented a significantly lower number of positive cells for this DNA oxidative maker than cartilage from the control group (Fig. 6c). In a similar way, we also detected the presence of 4-HNE in the cartilage of all experimental groups (Fig. 6b), although the levels of this end product of lipid peroxidation were significantly higher in the control group than those treated in the SW group (Fig. 6d).

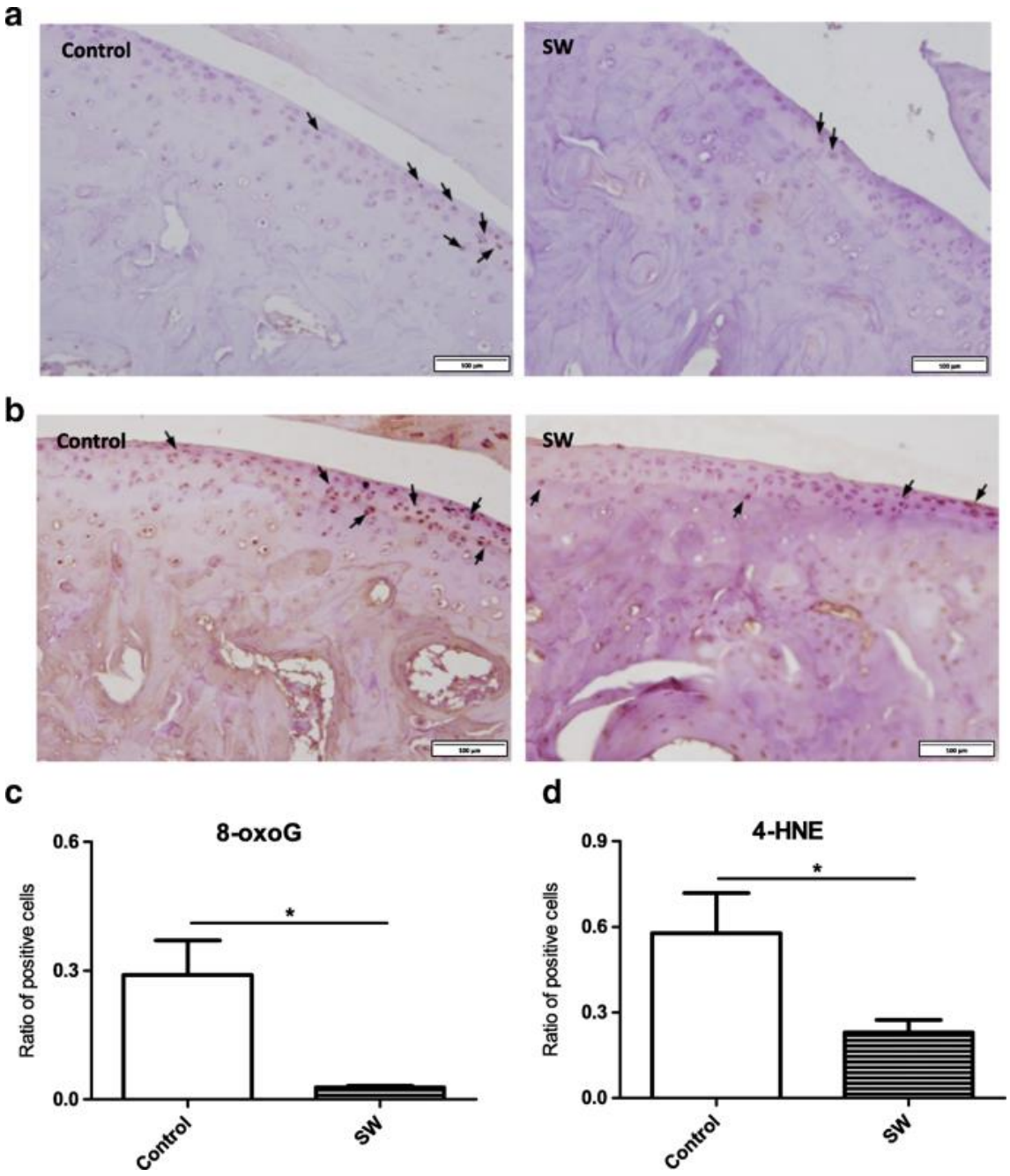

Fig. 6 Presence of oxidative damage markers in the articular cartilage. Representative samples of 8-hydroxy-2′-deoxyguanosine (8-oxo-dG) (**a**) and 4-hidroxy-2-nonenal (4-HNE) (**b**) immunohistochemistry in the cartilage from each group of study. Arrows indicate positive cells for immunostaining. Quantitative analysis of 8-oxo-dG- (**c**) and 4-HNE-positive (**d**) cells. Values are mean ± SEM ($n = 3$ independent animals for each condition). $^*P \leq 0.05$. Control, non-treated; SW, balneotherapy in sulfurous water

Discussion

OA treatment mainly involves drug intervention strategies in order to modulate symptoms and pathogenesis of this disease. However, available structure-modifying agents show limited efficacy and, in some cases, adverse effects (Matthews and Hunter 2011). Bath in water is habitually used as a complementary approach in the OA treatment that could favor the reduction of medication administration among other beneficial effects (Antonelli et al. 2018). However, the precise mechanisms of action of balneotherapy have not been completely clarified yet. In this study, we described for the first time to our knowledge the effect of balneotherapy in sulfurous water in an in vivo experimental model of OA. Our results suggest a protective effect of the balneological intervention on cartilage destruction and activation of oxidative pathways in the cartilage.

Orthopedic and joint goniometry studies showed non-conclusive findings, although results seemed to indicate that balneotherapy in sulfurous water reduces pain induced by surgical destabilization of the joint, as well as attenuate impaired motor function as detected by rotarod performance test. Similarly, Evcik et al. (2007) observed reduced pain and improved physical mobility in patients with knee OA after treatment with balneotherapy. We also detected that bath in sulfurous water may be more effective in terms of improved physical response than bath in tap water. In relation, previous findings showed that balneological interventions, such as hot mineral baths, better attenuate loss of quality of life in patients with OA than sham balneological interventions, i.e., tap water immersion (Yurtkuran et al. 2006; Güngen et al. 2012; Sarsan et al. 2012). These differences could likely be the results of a combination of factors by which thermal mineral waters activate mechanisms of action, such as mechanical, thermal, immunological, or chemical, that protect joint against development of rheumatic diseases (Fioravanti et al. 2011).

In this regard, the presence of therapeutically active components in the thermal mineral waters with specific chemo-physical properties could favor the activation of protective signaling pathways (Antonelli et al. 2018; Morer et al. 2017). Thus, extensive literature indicates therapeutic benefits of bath in sulfur-rich water in different pathologies like dermatitis (Lee and Wu 2014), stroke (Erceg-Rukavina and Stefanovski 2015), and articular diseases including rheumatoid arthritis (Santos et al. 2016) and osteoarthritis (Costantino et al. 2012; Kovács et al. 2012, 2016). The main active molecule in sulfurous and sulfated mineral-medicinal waters is H_2S (Gálvez et al. 2018), a gas that is primarily absorbed through lungs but could also enter in the organism through intact skin and subsequently exert its actions, although there is still scarce literature in this field (Fioravanti et al. 2011, 2017; National Research Council 2002). We and other authors have previously observed in vitro that this gas presents anti-oxidant and anti-inflammatory effects on IL-1β-activated chondrocytes (Burguera et al. 2014; Ha et al. 2015), likely through its capacity to attenuate pro-inflammatory signaling by inhibiting nuclear factor-κB (NF-κB) transcriptional activity and to promote nuclear factor E2-related factor 2 (Nrf2) signaling, master regulator of antioxidant, anti-inflammatory, and other cytoprotective mechanisms (Wallace and Wang 2015; Burguera et al. 2017). Besides, H_2S reduced destructive processes in the cartilage, downregulating the expression of enzymes involved in MEC degradation like MMP-3 and MMP-13 (Pan et al. 2016; Vela-Anero et al. 2017). Interestingly, we observed in the current study that balneotherapy in sulfurous water, but not in tap water, attenuated cartilage destruction, likely as a consequence of reduced MMP-13 levels detected in the joints from this treated group.

Surgically induced OA also elicited a detectable pathological reaction in the synovial tissue, although it only achieved a low grade of synovitis accordingly to the grading system of Krenn (Krenn et al. 2006). However, balneotherapy in sulfurous water failed to modulate the response in the synovial tissue. Conversely, previous studies observed that H_2S plays anti-inflammatory effects on fibroblast-like synoviocytes from OA and rheumatoid arthritis patients (Sieghart et al. 2015; Kloesch et al. 2010; Wu et al. 2016), alleviating, for instance, the inflammatory expression activated by IL-1 in these cells (Sieghart et al. 2015). Nevertheless, in agreement with our results, Tékus et al. (2018) observed in a chemical-induced OA that balneotherapy in thermal water rich in H_2S showed smaller synovial swelling, reduced number of fibroblasts, and less collagen, but there were no statistically significant differences in comparison with control. A possible explanation for the discrepancy between the effect of H_2S in in vitro and in vivo OA models could reside in the moment that histological analysis of synovium was performed, i.e., last stage of the experimental OA when synovial inflammation is likely into remission and destructive processes are taking place in the joint, so that differences between treated and control conditions in terms of synovitis are already indistinguishable. Likewise, Cozzi et al. (2015) detected in psoriatic arthritis patients treated with TNF inhibitors that mud bath therapy decreased synovial

inflammation analyzed by contrast-enhanced ultrasound, a reliable technique to detect and measure inflammation in synovial tissue.

Oxidative stress has been shown to play a contributory role in the pathogenesis of OA (Collins et al. 2018, Lepetsos and Papavassiliou 2016), mainly by causing damage to proteins, lipids, and DNA. 4-HNE and 8-oxo-dG are well-characterized oxidative damage makers that have been associated to pathological processes like mitochondrial dysfunction, apoptosis, mutagenesis, and inflammation in the joint (Abusarah et al. 2017; Biniecka et al. 2011a, b; Vaamonde-García et al. 2017). Besides, different in vitro studies show that oxidative stress is involved in altered chondrocyte survival (Cheleschi et al. 2017; Goutas et al. 2018; Lu et al. 2018) and act as second messengers in signaling cascades that activate the expression of catabolic mediators such as metalloproteinases like MMP-13 (Del Carlo et al. 2007; Gao et al. 2018), favoring cartilage degradation. We and other authors have previously described in vitro an anti-oxidant role of H_2S in chondrocytes (Burguera et al. 2014; Fox et al. 2012). In the current study, cartilage from balneotherapy in sulfur-rich water showed diminished levels of both oxidative markers after experimental OA. Altogether, our results suggest that balneotherapy could attenuate oxidative stress-activated signaling upregulating MMP-13 expression, in turn protecting cartilage against degradation. In relation, it has recently been described that spa therapy with balneotherapy exerts antioxidant and protective effects in patients with rheumatoid arthritis due to enhancement of antioxidant concentrations (Karagülle et al. 2017), such as glutathione peroxidase and coenzyme Q10 (Bajgai et al. 2017; Gvozdjakova et al. 2014). However, future studies are warranted to better explore the role of antioxidant actions of balneotherapy on its protective effects in the cartilage.

To sum up, our results indicate a beneficial effect of balneotherapy on experimental OA. Bath in sulfur-rich water seems to promote attenuation of cartilage destruction and oxidative damage after joint destabilization. In accordance, a previous study in OA patients showed reduced oxidative, inflammatory, and degradative biomarkers under sulfur-based spa therapies (Benedetti et al. 2010). Taken together, our findings reinforce the use of balneological intervention with sulfurous water as a complementary approach in the treatment of osteoarthritis. Likewise, a recent study in patients with hip osteoarthritis suggested that the combination of balneotherapy and exercise therapy achieves more sustained improvement of joint function and decreases in pain than exercise therapy alone (Kovács et al. 2016)

Conclusions

Although great number of studies highlight the importance of balneological interventions in secondary prevention and in the amelioration of OA symptoms (Antonelli et al. 2018; Fraioli et al. 2018), scientific evidence supporting its application in clinical practice is still scarce. The findings presented in the current study further advice the use of balneotherapy in sulfur-rich water on osteoarthritis, suggesting a protective effect on activation of oxidative and catabolic processes in the cartilage. Nevertheless, future clinical trials should be encouraged to test the effectiveness of balneotherapy in sulfurous water in OA patients.

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Ethics declarations
All animal experiments were performed according to protocols approved by the Local Ethical Committee of Animal Experimentation and European Directive 2010/63.

Conflict of Interest
The authors declare that they have no conflict of interest.

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